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## INTEGRIN $\alpha 6$ CLEAVAGE: A NOVEL MODIFICATION TO MODULATE CELL MIGRATION

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### Abstract

Integrins play a major role in cell adhesion and migration. Previous work reported that a cleaved form of integrin  $\alpha 6$  ( $\alpha 6p$ ) was detected in invasive human prostate cancer tissue, absent in normal prostate tissue and was produced by urokinase-type Plasminogen Activator (uPA) in a plasmin-independent manner. Using site-directed mutagenesis we identified amino acid residues R594 and R595, located in the “stalk” region of integrin  $\alpha 6$ , as essential for cleavage. The cleavage site is located on the extracellular region of the protein between the  $\beta$ -barrel domain and the thigh domain. Prostate cancer cells (PC3N) were stably transfected to over express the cleavable, wild type (PC3N- $\alpha 6$ -WT) or the non-cleavable form of integrin  $\alpha 6$  (PC3N- $\alpha 6$ -RR). The number of cells invading laminin 111 and laminin 332 coated filters by PC3N- $\alpha 6$ -WT cells increased by three fold as compared to PC3N- $\alpha 6$ -RR cells. Plasminogen Activator Inhibitor-1 (PAI-1) reduced the invasion of PC3N- $\alpha 6$ -WT cells by approximately 42% through laminin 332 coated filters and plasmin inhibitor aprotinin had no significant effect. Linear cell migration increased production of integrin  $\alpha 6p$  in the PC3N- $\alpha 6$ -WT cells and not in the PC3N- $\alpha 6$ -RR cells and 32% of the PC3N- $\alpha 6$ -WT cells migrated on laminin 111 in the linear migration assay as compared to the 5% PC3N- $\alpha 6$ -RR cells. These data taken together suggest that the uPA mediated cell surface cleavage of the  $\alpha 6$  integrin extracellular domain is involved in tumor cell invasion and migration on laminin.

### Keywords

Integrin  $\alpha 6$ ; cleavage; prostate cancer; ECM; migration; laminin; uPA; urokinase

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## INTRODUCTION

Integrins are heterodimeric cell surface proteins composed of an  $\alpha$  and a  $\beta$  subunit. Each  $\alpha\beta$  combination has its own binding specificity and signaling properties [1]. Different integrin combinations act as receptors to different extracellular matrix (ECM) proteins [1,2]. They are involved in many processes including cell migration, cell adhesion, differentiation, blood clotting, tissue organization and cell growth as well as cancer cell migration, invasion and metastasis. Integrins generally contain a large extracellular domain ( $\alpha$  subunit ~1000 residues and  $\beta$  subunit ~750 residues), a transmembrane domain and a short cytoplasmic domain (~50 residues or less) with the exception of  $\beta 4$  whose cytoplasmic domain is large (more than 1000 residues) [3,4].

The structure of the extracellular segment of the  $\alpha v$  integrin subunit has been determined using protein crystals of soluble  $\alpha v\beta 3$  integrin fragment [5]. The amino terminus of the  $\alpha v$  integrin subunit contains a seven-bladed  $\beta$ -propeller structure [6]. It is followed by a tail region composed of three  $\beta$ -sandwiched domains: an Ig-like “thigh” domain and two very similar domains that form the “calf” module [6]. It has been shown that the tail region can fold back at ~135 degree angle forming a V-shaped structure with a “genu” between the thigh domain and the calf module [6].

Our previous work has shown that a structural variant of the  $\alpha 6$  integrin called the  $\alpha 6p$  exists that is missing over half of the extracellular segment, the presence of which has also been reported by others [7,8]. This variant is present in prostate cancer tissue but is absent in normal prostate tissue [9]. The  $\alpha 6p$  is missing the extracellular  $\beta$ -propeller domain associated with ligand binding but remains bound to its  $\beta$  integrin partner [7]. We have also shown using four different experimental approaches that  $\alpha 6p$  is produced by proteolytic cleavage of the  $\alpha 6$  integrin by Urokinase-type plasminogen Activator (uPA) in a plasmin-independent manner [9]. The  $\alpha 6p$  integrin is a 70 kDa protein, and mass spectrometry analysis has shown that the amino terminal end of the molecule contains amino acids starting at arginine 596 [7]. Using a multiple sequence alignment tool, this position in the  $\alpha 6$  integrin has been shown to lie within an accessible loop upstream from the genu region described for the  $\alpha v$  integrin subunit [5].

The protease that cleaves the  $\alpha 6$  integrin, uPA, is a secreted 54-kDa serine protease which cleaves plasminogen as a primary substrate [10]. uPA has also been shown to catalyze the proteolytic cleavage of the extracellular matrix protein fibronectin [11], hepatocyte growth factor/scatter factor (HGF/SF) [12] and macrophage-stimulating protein (MSP) [13].

The aim of this study was twofold: first, to use site directed mutagenesis to determine the amino acid sequences necessary for the uPA dependent cleavage of the  $\alpha 6$  integrin. Second, to determine the phenotypic properties of cells expressing the non-cleavable form of the integrin  $\alpha 6$ . Our results indicate that integrin  $\alpha 6$  cleavage is a dynamic feature contributing to cell invasion and migration on laminin.

## MATERIALS AND METHODS

### Cells

All cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. K562, PC3N and HaCaT cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL, MD, USA) plus 10% fetal bovine serum (FBS). The wildtype (WT) and mutated (RR) integrin  $\alpha 6$  transfected cell lines K562- $\alpha 6$ -WT, K562- $\alpha 6$ -RR, PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR were grown in IMDM plus 10% FBS in presence of 6 $\mu$ g/ml Blasticidin and 1  $\mu$ g/ml Zeocin (Invitrogen Corp., CA, USA). These cells were induced using 0.02 $\mu$ g/ml Doxycycline (Sigma,

St. Louis, MO, USA.). The PC3N cells are a variant of the PC3 prostate carcinoma cell line [14].

### Antibodies and chemicals used

Anti- $\alpha 6$  integrin antibodies include: J1B5, rat monoclonal was a generous gift from Dr. Caroline Damsky (University of California, San Francisco, USA) [15] and AA6A rabbit polyclonal antibody was produced by Bethyl Laboratories Inc. (Montgomery, TX, USA). AA6A is specific for the last 16 amino acids (CIHAQPSDKERLTSDA) in the cytoplasmic region of the human  $\alpha 6A$  sequence [16]. The monoclonal  $\beta 1$  integrin antibody, TS2/16 [17] was produced by the hybridoma cell line obtained from the American Type Culture Collection. The rabbit anti-actin antibody, AAN01 was obtained from Cytoskeleton, Denver, CO, U.S.A. The monoclonal antibody against uPAR was purchased from American Diagnostica, Greenwich, CT, U.S.A. Rabbit anti-uPA antibody was purchased from Abcam (Cambridge, MA, U.S.A.). Urokinase was purchased from Chemicon, Temecula, CA, USA and consisted of a mixture of single chain and double chain forms of uPA. Human recombinant EGF was purchased from Invitrogen Corp., Carlsbad, CA, U.S.A. Plasminogen Activator Inhibitor-1 was from Calbiochem, San Diego, CA, U.S.A. Aprotinin was purchased from Sigma-Aldrich, St. Louis, MO, U.S.A.

### Site-directed mutagenesis

The cDNA for wild-type  $\alpha 6A$  was a generous gift from Dr. Isaac Rabinovitz. After cloning it into the pcDNA4/TO vector we used different sets of primers to induce the desired mutations in the  $\alpha 6$  integrin. We used QuikChange Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek Texas). The primers were designed using the recommendations of the manufacturer and were:

Primers for RR----> AA mutation 5'ggagatccaagagccaagctctGCTGCGegagtgaattcacttccagaag 3' 5'CttctggaagtgaattcactcgcGCaGCagagcttgctcttggatctcc 3' Primers for RV-----> AA mutation 5'gccaagagccaagctctcgttagggcagcgaattcacttccagaagttctcc3' 5' GGAAGAAGTTCTGGAAGTGAATTCGCTGCCCTACGAGAGCTTGCTCTTGGC3' Primers for R536-----> A mutation 5' AAAATCTGGGCTATCCTCAGCAGTTCAGTTTCGAAACCAAG3' 5' CTTGGTTTCGAAACTGAACTGCTGAGGATAGCCAGATTTT3' Primers for R594-----> A mutation 5'GATCCAAGAGCCAAGCTCTGCAAGGCGAGTGAATTCACCTC3' 5' GAAGTGAATTCACCTCGCCTTGCAGAGCTTGGCTCTTGGATC3' Primers for R595-----> A mutation 5'GAGCCAAGCTCTCGTGCGGAGTGAATTCACCTCC3' 5' GGAAGTGAATTCACCTCGCGCACGAGAGCTTGGCTC3'

The reactions were carried out as recommended by the manufacturer. The K562 cells were transfected by electroporation as described previously [18]. Briefly, 30 $\mu$ g of plasmid and 10 $\mu$ g of repressor plasmid (pcDNA6/TR) were added to 10 million cells in a final volume of 800 $\mu$ l of serum free RPMI media in a 0.4cm electroporation cuvette. The cells were electroporated at 960 microfarads and 320 V. After 10 minutes on ice the cells were plated in IMDM media with 10% serum for 24 hours. The media was replaced with fresh media containing 0.2 $\mu$ g/ml doxycycline and the cells were incubated for another 24 hours. The cells were then analyzed for  $\alpha 6$  integrin cleavage by immunoprecipitating the  $\alpha 6$  integrin with J1B5 and adding 20 $\mu$ g of uPA in the immunocomplexes for 3 hours on ice. The samples were analyzed by immunoblotting using the anti- $\alpha 6$  integrin rabbit polyclonal antibody, AA6A. K562 stably transfected cells (K562- $\alpha 6$ -WT expressing wildtype integrin  $\alpha 6$  and K562- $\alpha 6$ -RR expressing the non-cleavable integrin  $\alpha 6$ ) were selected using the rat monoclonal anti-integrin  $\alpha 6$  antibody J1B5 and magnetic microbeads (MACS, Miltenyi Biotech, Auburn, CA, USA) as per the manufacturer's instructions. The PC3N cells were transfected using Effectene (Qiagen,

Valencia, CA, U.S.A.) using the recommendations of the manufacturer. The stable clones (PC3N- $\alpha 6$ -WT expressing wildtype integrin  $\alpha 6$  and PC3N- $\alpha 6$ -RR expressing the non-cleavable integrin  $\alpha 6$ ) were isolated after treatments with 6  $\mu\text{g}/\text{ml}$  Blastidicin and 1  $\mu\text{g}/\text{ml}$  Zeocin (Invitrogen Corp., CA, USA).

### Flow cytometry

Flow cytometric analysis was carried out as described earlier[19]. Briefly, K562, K562- $\alpha 6$ -WT and K562- $\alpha 6$ -RR cells were washed in PBS and incubated with rat anti-integrin  $\alpha 6$  antibody J1B5 (1:10) for 30 min at 4 °C, washed two times with PBS + 1% BSA + 10mM Sodium Azide. The cells were then incubated with secondary anti-rat antibody Alexa 488 for 30 min at 4 deg C in the dark, washed two times with PBS + 1% BSA + 10mM Sodium Azide and pellet suspended in 1% Formaldehyde solution and analyzed on the BD FACScan (BD Biosciences, San Jose, CA USA).

### Immunoprecipitations

Cells were washed three times with HEPES buffer and lysed in cold RIPA buffer (150 mM NaCl, 50 mM Tris (Biorad, Hercules, CA), 5 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) SDS, pH 7.5) plus protease inhibitors inhibitors (PMSF, 2 mM; leupeptin and aprotinin, 1  $\mu\text{g}/\text{ml}$ ). The lysate was briefly sonicated on ice and precleared by rotating it with 50 $\mu\text{l}$  of protein G sepharose beads at 4 °C for 1 hour. The beads were removed by centrifugation, and the supernatant was collected for immunoprecipitations. Each reaction mixture contained 35  $\mu\text{l}$  of protein G sepharose and 5 $\mu\text{g}$  of antibody. Anti-integrin  $\alpha 6$  rat monoclonal antibody J1B5, mouse anti-integrin  $\beta 1$  antibody TS216 and mouse anti-uPAR antibody were used. The final volume of the lysate was adjusted to 500  $\mu\text{l}$  with RIPA buffer. The mixture was rotated for 18 hours at 4 °C and complexes were then washed four times with cold RIPA buffer. The pellet was dissolved in 2X non-reducing sample buffer and boiled for 5 minutes. The samples were analyzed by immunoblotting procedures.

### Immunoblotting

Cells were lysed in RIPA and the resulting protein solubilized in non-reducing sample buffer and analyzed by a 10 % SDS-PAGE gel. Proteins resolved in the gel were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), incubated with anti-integrin  $\alpha 6$  antibody AA6A and detected using a secondary antibody conjugated to horseradish peroxidase. Rabbit anti-uPA antibody was used to probe for uPA. Membranes were probed with the anti-actin rabbit polyclonal antibody from Cytoskeleton (Denver, CO, USA) as loading control. Visualization was by chemiluminescence (ECL Western Blotting Detection System, Amersham, IL, USA).

### Integrin $\alpha 6$ model

The homology model of the human integrin  $\alpha 6$  was created using Modeller (version 7) maintained at the California Institute for Quantitative Biomedical Research, UCSF, San Francisco, CA, USA. The model is based on the crystal structure of integrin  $\alpha \nu \beta 3$  (pdb:1JV2). Energy minimization was done using Sybyl 7.0 (Tripos Inc. St. Louis, MO, USA.).

### Transwell cell migration assay

Cell invasion studies were done in 24-well transwell cell culture chambers with the upper chamber containing filters of 8.0- $\mu\text{m}$  pore size (BD Falcon Cell Culture Inserts, 8.0  $\mu\text{m}$  24 well and BD Falcon Cell Culture Companion Plates for Inserts, 24 well, from BD, NJ, U.S.A.). The upper surface of the filters was coated with a different matrices including 20  $\mu\text{g}/\text{ml}$  laminin 111, 40  $\mu\text{g}/\text{ml}$  fibronectin (BD Biosciences, NJ, U.S.A.), 40  $\mu\text{g}/\text{ml}$  collagen IV (Gibco, BRL, MD, USA) or laminin 332 (HaCaT cell serum free conditioned media) and dried overnight at

RT. Approximately 50,000 cells were seeded on the upper chamber wells together with or without 3  $\mu\text{g/ml}$  PAI-1 or 10  $\mu\text{g/ml}$  aprotinin in serum free conditions. 1 ng/ml EGF was placed in the lower chamber as a chemo-attractant. After 24h of incubation at 37 °C, cells that invaded into the lower compartment were stained with 0.5% Crystal Violet/20% methanol and counted. All experiments were done twice in triplicate.

### Migration Assay

Doxycycline induced cells were grown to confluency on 20  $\mu\text{g/ml}$  laminin 111, 40  $\mu\text{g/ml}$  fibronectin (BD Biosciences, NJ, U.S.A.), 40  $\mu\text{g/ml}$  collagen IV (Gibco, BRL, MD, USA) or laminin 332 (HaCaT cell conditioned media) coated square coverslips in IMDM (Gibco BRL, MD, USA) plus 1% fetal bovine serum (FBS). The source of laminin 332 was serum free conditioned medium from HaCaT cells and contains the pro-migratory form of laminin 322 (data not shown). A scratch was made diagonally across the square coverslip with a plastic cell scraper (Fisherbrand Cat. # 08-773-2). The coverslips were then rinsed in sterile PBS and placed in fresh serum free IMDM medium containing the different ligands (laminin 111, laminin 322, fibronectin or collagen IV) and 1 ng/ml EGF to induce migration. PAI-1 (3  $\mu\text{g/ml}$ ) was used to inhibit uPA activity in the migration assay. The cells were incubated for 3, 6, 9 or 12 hours. The cells were fixed with cold methanol for 10 min followed by cold acetone. On drying, the cells were then stained with 0.5  $\mu\text{g/ml}$  DAPI for 10 min. The coverslips were washed in PBS, post-fixed in Ethanol for 4 min and mounted using Prolong Antifade (Molecular Probes, Or, USA). The cells were observed using a Zeiss Axiovert microscope (10X magnification) and images were collected using a CCD camera. Quantification was done using the particle counting feature of Scion Image Software.

## RESULTS AND DISCUSSION

### The uPA mediated $\alpha 6$ integrin cleavage required amino acid residues R594 and R595

Site-directed mutagenesis was performed on selected amino acid residues that were identified as candidates for the cleavage site. These residues were selected on the basis of our previous work in which MALDI mass spectrometry and liquid chromatography-tandem mass spectrometry identified ten non-continuous amino acid fragments within the  $\alpha 6$ p variant corresponding exactly to the predicted trypsin fragments coded by exons 13–25 of the  $\alpha 6$  integrin sequence [7]. Further, the sequencing data confirmed that both the heavy and light chains of the  $\alpha 6$ p variant contained identical portions of the full-length  $\alpha 6$  integrin and that  $\alpha 6$ p contained at least amino acid R596. This information coupled with the knowledge that the protease that cleaves the  $\alpha 6$  integrin is uPA, led us to mutate different arginine residues upstream from R596 (Fig. 1A). We excluded sequences that were conserved between the  $\alpha 6$  integrin and the non-cleavable  $\alpha 3$  integrin. We first mutated residues R536, R594, R595, followed by inducing double mutations R594R595 and R596V597. All residues were mutated to alanine. The constructs were transfected into an  $\alpha 6$  null erythroleukemia cell line K562 resulting in K562- $\alpha 6$ -WT cells expressing the wild type integrin  $\alpha 6$  and the K562- $\alpha 6$ -RR cells expressing the mutated, non-cleavable form of integrin  $\alpha 6$ . After immunoprecipitation of the mutated or wild type  $\alpha 6$  integrin, uPA, which consisted of a mixture of single chain and double chain form [20], was added to the immunocomplexes and the samples were analyzed by SDS-PAGE analysis followed by western blotting for the  $\alpha 6$  integrin (Fig. 1A). The data showed that mutation of arginine 594 to alanine significantly reduced the ability of uPA to cleave the  $\alpha 6$  integrin as compared to the wild-type  $\alpha 6$  or other mutants (R536-A and R596V597-AA). Interestingly mutation of both arginine residues 594 and 595 was required to completely abolish the cleavage of the  $\alpha 6$  integrin by uPA.

It was possible that ectopic expression of the mutant integrin could occur without correct localization or heterodimerization. The presence of the mutant and wild type integrin  $\alpha 6$  on



the cell surface of the K562- $\alpha 6$ -RR and K562- $\alpha 6$ -WT stably transfected cell lines and the parental K562 cells was analyzed using flow cytometry. As expected, integrin  $\alpha 6$  was not detected in the parental K562 cells, however a significant amount of fluorescence was recorded in the K562- $\alpha 6$ -WT and K562- $\alpha 6$ -RR cells indicating the presence of integrin  $\alpha 6$  on the cell surface (Fig. 1B). We next determined if the mutant and the wild-type integrin  $\alpha 6$  expressed in the K562- $\alpha 6$ -RR and K562- $\alpha 6$ -WT cells were able to pair with the  $\beta 1$  subunit that is constitutively expressed in the K562 cells. Immunoprecipitations (IPs) were performed on doxycycline induced K562- $\alpha 6$ -RR and K562- $\alpha 6$ -WT cells using anti-integrin  $\alpha 6$  and anti-integrin  $\beta 1$  antibodies and analyzed by western blotting to detect the  $\alpha 6$  integrin. IP of the cell lysates with either  $\alpha 6$  or  $\beta 1$  integrin antibodies will retrieve the  $\alpha 6$  integrin. The results indicated that both the wild type and mutant  $\alpha 6$  interacted with the  $\beta 1$  subunit (Fig. 1C). The data further indicates that the R594 R595 to A594 A595 substitution does not affect the ability of the integrin to bind to its  $\beta$  partner or become resident on the cell surface.

In order to spatially localize R594 and R595 on the  $\alpha 6$  integrin, we created a homology model based upon the  $\alpha v\beta 3$  integrin (Fig. 2A). The crystal structure of  $\alpha v\beta 3$  is known [5]. A model of integrin  $\alpha 6$  was created using integrin  $\alpha v$  structure as a template (Fig. 2A). The model spans residues 24–1004, consisting of the calf-1, calf-2 and genu region (shown in black) and the thigh and the  $\beta$ -propeller domain (shown in white). Integrin  $\alpha 6$  undergoes uPA-mediated cleavage at the RR site, which is located at the beginning of the thigh region (Fig. 2A). The location of this site in the model indicated that the majority of the thigh region and the entire  $\beta$ -propeller region were removed. The integrin  $\alpha 6p$  contains calf-1, calf-2, genu and a segment of the thigh region. The uPA mediated cleavage site within the two arginine residues is potentially accessible for cleavage, as it lies exposed in the outer region of integrin  $\alpha 6$  as seen in Fig. 2A.

### The cleavage residues used by uPA are found within human, mouse and rat integrin $\alpha 6$

To determine if the cleavage residues used by uPA on the human integrin  $\alpha 6$  were present in integrin  $\alpha 6$  from other species, we aligned sequences from rat, mouse, chicken, worm and fly. The results indicate that the RR sequence was identical in human, rat and mouse integrin  $\alpha 6$  (Fig. 2B). The RR sequence was not found in the integrin  $\alpha$  chain sequence of *C. elegans* and the S40311 integrin sequence of *D. melanogaster* (Fig. 2B). The rat and mouse integrin  $\alpha 6$  contains both the R594 and R595 residues present in human integrin  $\alpha 6$ , suggesting that rat and mouse integrin  $\alpha 6$  would undergo uPA-mediated cleavage. Simplified model systems may be useful to study the physiological effects of uPA-mediated cleavage of integrin  $\alpha 6$  to  $\alpha 6p$  in vivo.

Integrins  $\alpha 3$ ,  $\alpha 6$  and  $\alpha 7$  are laminin receptors. Integrin  $\alpha 3$  and  $\alpha 6$  are found predominantly in epithelial cells and integrin  $\alpha 7$  is found predominantly in skeletal and cardiac muscles and neural cells [21,22]. Our earlier work has shown that uPA does not cleave integrin  $\alpha 3$  [9]. Comparison of the cleavage site residues between integrin  $\alpha 6$  and integrin  $\alpha 3$  shows the absence of the RR sequence in integrin  $\alpha 3$  (Fig. 2C). Integrin  $\alpha 7$  does have the two arginine residues required for the uPA-mediated cleavage but is not expressed in epithelial cells [21]. It remains to be determined whether integrin  $\alpha 7$  is processed on the cell surface by uPA.

### Human prostate cancer cell lines expressed cleavable and non-cleavable integrin $\alpha 6$

The cleaved form of integrin  $\alpha 6$ ,  $\alpha 6p$ , has been observed in human prostate cancer specimens and not in the adjacent normal human prostate tissue [9]. In order to study the biological significance of this cleavage, we transfected prostate cancer cell line PC3N with wild type cleavable integrin  $\alpha 6$  generating the PC3N- $\alpha 6$ -WT. We also transfected PC3N cells with the mutated non-cleavable integrin  $\alpha 6$  in which the residues R594 and R595 were substituted by residues A594 and A595 resulting in the generation of the PC3N- $\alpha 6$ -RR cell line. These cells

over express the wild type or the mutated integrin  $\alpha 6$  respectively, in presence of doxycycline, to equivalent levels as estimated by western blot (Fig. 3A). It should be noted that in the absence of doxycycline, both the PC3N- $\alpha 6$ -WT and the PC3N- $\alpha 6$ -RR cells contain similar levels of endogenous integrin  $\alpha 6$ .

In order to determine if the transfected wild type and mutated integrin  $\alpha 6$  could pair with the endogenous integrin  $\beta 1$ , immunoprecipitations were performed on doxycycline induced PC3N- $\alpha 6$ -RR and PC3N- $\alpha 6$ -WT cells using anti-integrin  $\alpha 6$  or  $\beta 1$  antibodies and analyzed by western blotting to detect the  $\alpha 6$  integrin. The results indicated that both the induced wild type and mutant  $\alpha 6$  interacted with the  $\beta 1$  subunit (Fig. 3B).

We next tested the ability of exogenously supplied uPA to cleave the induced integrin forms. In the presence of doxycycline, PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR cells were treated with 20 $\mu$ g/ml uPA for up to 120 min. Consistently increasing levels of integrin  $\alpha 6$ p, from 0 time to 120 min, in the PC3N- $\alpha 6$ -WT cells were observed. The increase in integrin  $\alpha 6$ p levels was observed with a corresponding decrease in the full-length integrin  $\alpha 6$  levels (Fig. 4A). In the PC3N- $\alpha 6$ -RR cells, however, no significant formation of integrin  $\alpha 6$ p was seen (Fig. 4B). The faint bands that are visible in the blot are due to  $\alpha 6$ p generated from endogenous expression of the wild type integrin  $\alpha 6$  (Fig. 4B). The induced wild type integrin  $\alpha 6$  was cleavable by exogenously supplied uPA in a time dependent manner (Fig. 4B). A majority of the  $\alpha 6$ -WT integrin is cleavable by uPA. The  $\alpha 6$ -WT that is not cleavable by uPA is either protected from cleavage while resident on the cell surface or based upon our previous findings, has not yet reached the cell surface (7). The mutant integrin was found to be refractory to uPA mediated cleavage. The data shows that the mutation due to the change from the arginine residues (R594, R595) to alanine residues prevents uPA-mediated cleavage of integrin  $\alpha 6$  to  $\alpha 6$ p (Fig. 4B).

### Blocking integrin $\alpha 6$ cleavage reduces cell invasion and migration

Several previous studies have implicated uPA in tumor cell invasion [23–26]. We next determined the influence of the cleavage of integrin  $\alpha 6$  to  $\alpha 6$ p on cell invasion. PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR cells were placed on laminin 111, fibronectin, collagen IV or laminin 332 coated and uncoated 8.0  $\mu$ m pore filters and 1 ng/ml EGF was placed in the lower wells to serve as a chemo-attractant in serum free media. The cells were incubated 24 hours and the number of cells that had invaded through the filter per microscopic field was quantified. The results are shown in Fig. 5A. Minimal, if any, invasion was observed on uncoated and collagen IV coated filters. Although some PC3N- $\alpha 6$ -WT as well as PC3N- $\alpha 6$ -RR cells invaded the fibronectin and laminin 111 coated filters, maximum invasion was observed on Laminin 332 coated filters. Approximately 84 cells per microscopic field of PC3N- $\alpha 6$ -WT cells had invaded the filters as compared to 26 cells per microscopic field of PC3N- $\alpha 6$ -RR (Fig. 5A).

In order to determine the effect of the uPA inhibitor PAI-1 on the invading cells, the cells were placed on laminin 111 or laminin 332 coated filters and incubated with 3  $\mu$ g/ml PAI-1. Consistent with the results in Fig. 5A, laminin 332 coated filters provided the most effective support for invasion by either cell line. The uPA inhibitor PAI-1, inhibited invasion of laminin 332 coated filters by 42% and 45% in the PC3N- $\alpha 6$ -WT and the PC3N- $\alpha 6$ -RR cells respectively (Fig. 5B). We demonstrated in earlier work that uPA cleaves integrin  $\alpha 6$  in a plasmin independent manner [9]. However, in order to determine whether membrane bound plasmin affects the integrin cleavage, the cells were incubated with a plasmin inhibitor, aprotinin [27, 28]. The addition of aprotinin to the cells did not significantly reduce PC3N- $\alpha 6$ -WT or PC3N- $\alpha 6$ -RR cell invasion on laminin 111 or laminin 332 coated filters (Fig. 5B), indicating that the observed invasion is independent of membrane bound plasmin.

Previous studies have shown that TPA (12-O-Tetradecanoyl-phorbol 13-Acetate) induces cell migration in migration assays [29]. Our earlier work has shown that the cleavage of integrin

$\alpha 6$  to  $\alpha 6p$  can be induced by TPA in a uPA mediated manner [9]. Further, uPA has been shown to be present at the leading edge in wound healing experiments [30,31]. These observations suggest that the cleavage of the  $\alpha 6$  integrin may play a role in cell migration. In order to determine the significance of the cleavage of integrin  $\alpha 6$  to  $\alpha 6p$  on linear cell migration, PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR cells were grown on a laminin 111, fibronectin, collagen IV and laminin 332 coated coverslips. Migration was induced by scratching [32] and EGF stimulation in the absence of serum in the media. After 12 hours of incubation, the images of the cells entering into the scratch on either collagen IV, fibronectin, laminin 111 or laminin 332 were collected (Fig. 6A). The quantification of the images are shown in Fig. 6B. In this assay, the greatest migration by the PC3N- $\alpha 6$ -WT cells was observed on laminin 111 (32%) followed by fibronectin (12%) and laminin 332 (10%), with virtually no migration (approximately 4%) observed on collagen IV. In particular, the laminin 111 coated coverslips resulted in the most migration with approximately 32% of the PC3N- $\alpha 6$ -WT cells entering the scratch whereas approximately 5% of the PC3N- $\alpha 6$ -RR cells were observed (Fig. 6A, B). These data indicate that by comparing the best migration promoting ligand in this assay (i.e. laminin 111), there was approximately 6.4 fold difference between the number of PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR cells migrating into the scratch. It should be noted that the mutant cells as compared to the wild type cells also were hindered in migrating on fibronectin (approximately 2.4 fold difference) and laminin 332 (1.4 fold difference) with no significant difference on collagen IV (Fig. 6B). We next tested if blocking uPA activity would inhibit migration on the best migrating promoting ligand, laminin 111. The cells were induced to migrate by generating the scratch and 3  $\mu$ g/ml PAI-1 was added. Only 2% PC3N- $\alpha 6$ -WT cells migrated into the scratch in the presence of PAI-1 as compared to 32% in absence of PAI-1 (Fig. 6C). These observations indicate that uPA mediated cleavage of integrin  $\alpha 6$  to  $\alpha 6p$  facilitated migration on laminin 111.

It is interesting to note that independent of the migration type assay, the PC3N- $\alpha 6$ -WT are better equipped to migrate as compared to the PC3N- $\alpha 6$ -RR cells. However, it appears that the assays are ligand dependent, i.e. laminin 332 coated filters facilitate invasion whereas laminin 111 coated coverslips support linear cell migration of prostate cancer cells. Taken together, the data suggests that integrin  $\alpha 6$  cleavage assists cell invasion on laminin 332 and cell migration on laminin 111.

### Integrin $\alpha 6p$ is induced in migrating cells

Since the data suggested that the ability to cleave A6 integrin facilitated migration, we next determined if the A6 integrin was indeed cleaved under conditions of induced migration. The doxycycline induced PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR cells were analyzed for the presence of the integrin  $\alpha 6p$  during migration. An increase in integrin  $\alpha 6p$  levels was observed in the PC3N- $\alpha 6$ -WT cells 18 hours after induced migration. Comparable levels of full-length integrin  $\alpha 6$  were seen in the PC3N- $\alpha 6$ -WT cells with or without migration. As expected, a comparable low level of integrin  $\alpha 6p$  was observed in the mutant containing PC3N- $\alpha 6$ -RR cells with or without migration due to the cleavage of endogenous integrin  $\alpha 6$  (Fig. 7A). Densitometric analysis was carried out on the blot to determine if the ratio of the  $\alpha 6p$  signal to the full-length form was altered under conditions of induced migration (Fig. 7B). An increased ratio of  $\alpha 6p$  to  $\alpha 6$  integrin occurs in the PC3N- $\alpha 6$ -WT cells that have been induced to migrate as compared to the PC3N- $\alpha 6$ -WT that are not induced. The ratio of  $\alpha 6p$  to  $\alpha 6$  in the uncleavable mutants (PC3N- $\alpha 6$ -RR cells) was not influenced by the scratch procedure. We note with interest that integrin  $\alpha 6p$  appears as a doublet at approximately 75 kDa when migration is induced by scratching in the PC3N- $\alpha 6$ -WT cells (Fig. 7A). These two forms are presumed to represent altered levels of post-translational modifications like glycosylation and palmitoylation resulting in altered molecular weight [34–36]. We have previously reported that five of the nine putative glycosylation sites on integrin  $\alpha 6$  are retained on integrin  $\alpha 6p$  [7]. Hence the two forms of integrin  $\alpha 6p$  observed in the western blot may correspond to such modified integrins. It is



important to note that the acute cellular response to uPA treatments (Fig. 4) over a period up to 120 minutes, results in an increase in  $\alpha 6$ p levels that corresponds to a drop in the full length form of  $\alpha 6$  integrin. This is not observed if the analysis occurs after 18 hours, since enough time has elapsed for newly synthesized  $\alpha 6$ , not yet on the cell surface and not susceptible to cleavage, to be represented in the total full-length band.

Taken together, our data suggests that the integrin  $\alpha 6$  undergoes uPA-mediated cleavage at residues R594 R595 in a time dependent manner enhancing cell invasion and migration on laminin. Further understanding of factors regulating integrin cleavage holds promise for either modulating or detecting early tumor cell invasion or migration. Future work will be directed towards understanding the regulatory features dictating the cleavage of the integrin  $\alpha 6$  on the tumor cell surface.

#### Acknowledgements

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## The abbreviations used are

uPA

urokinase-type plasminogen activator

**PAI-1**

Plasminogen Activator Inhibitor –1

**EGF**

Epidermal Growth Factor

**SDS**

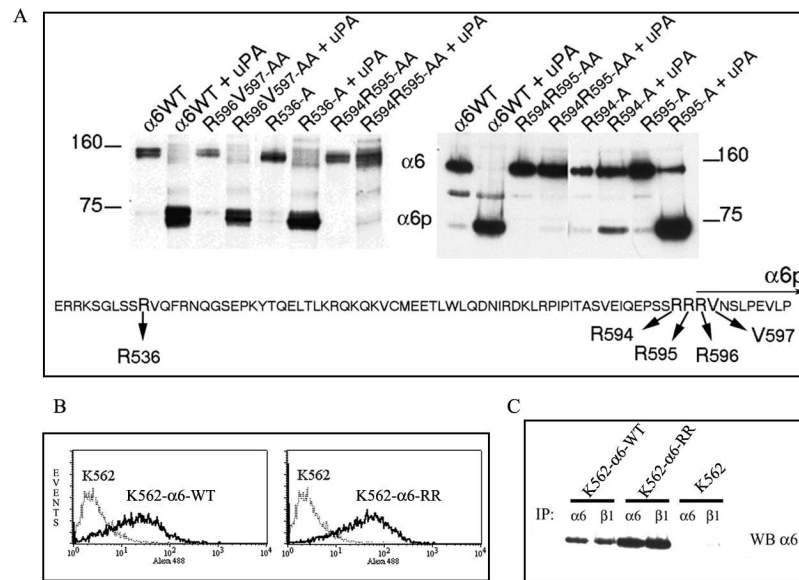
Sodium dodecyl sulphate

**PAGE**

Polyacrylamide gel electrophoresis

**DOX**

Doxycycline

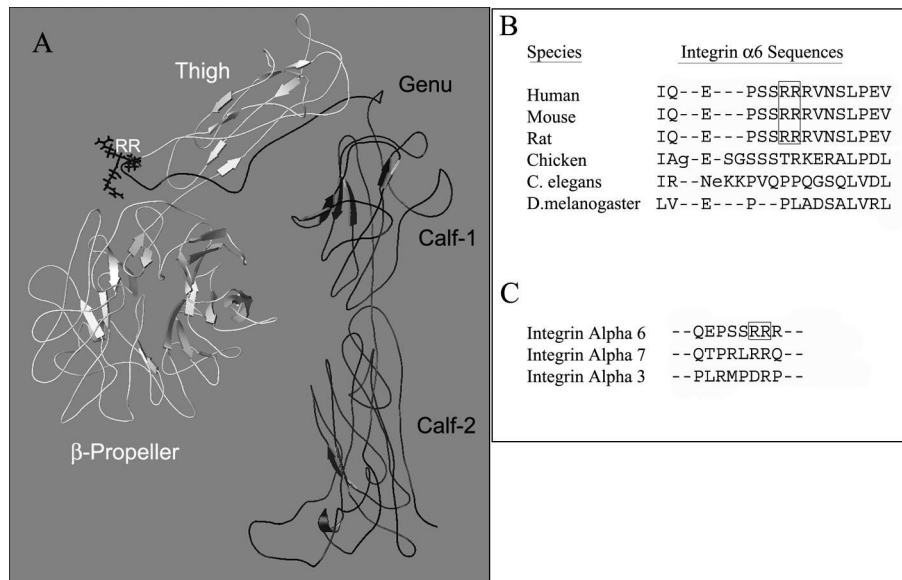


### Fig 1. Site-directed mutagenesis analysis of the $\alpha 6$ integrin

A. Five different residues (R536, R594, R595, R596 and V597) were replaced by alanine in the  $\alpha 6$  integrin extracellular domain. K562 cells were transfected with different mutated  $\alpha 6$  integrin constructs and the  $\alpha 6$  variants induced by Doxycycline. After 24 hours the  $\alpha 6$  integrin was immunoprecipitated from the cells and the immunocomplexes were treated with 20  $\mu\text{g}/\text{ml}$  uPA. The resulting  $\alpha 6$  and  $\alpha 6\text{p}$  integrin was detected by western blot analysis using the AA6A rabbit polyclonal antibody. A partial sequence of the integrin  $\alpha 6$  flanking the cleavage site is shown for illustrative purposes and the mutated residues are indicated with arrows. The horizontal arrow labeled  $\alpha 6\text{p}$  indicates the amino terminal end of the truncated integrin as analyzed by mass spectrometric analysis in our earlier work (7).

B. Surface expression of wild type and mutated integrin  $\alpha 6$  in transfected K562 cells by flowcytometry. Untransfected cells (K562-light line) or transfected cells (K562- $\alpha 6$ -WT or K562- $\alpha 6$ -RR - heavy lines) were treated with primary Rat anti-integrin  $\alpha 6$  antibody J1B5 followed by Alexa 488 anti-rat antibody and visualized using the BD FACScan.

C. The wild type and mutated integrin  $\alpha 6$  binds to integrin  $\beta 1$ . Immunoprecipitations (IP) of integrin  $\alpha 6$  and integrin  $\beta 1$  in doxycycline induced K562- $\alpha 6$ -WT and K562- $\alpha 6$ -RR and untransfected K562 using rat anti-integrin  $\alpha 6$  antibody J1B5 and mouse anti-integrin  $\beta 1$  TS2/16 were done. Rabbit anti-integrin  $\alpha 6$  antibody AA6A was used for western blotting.



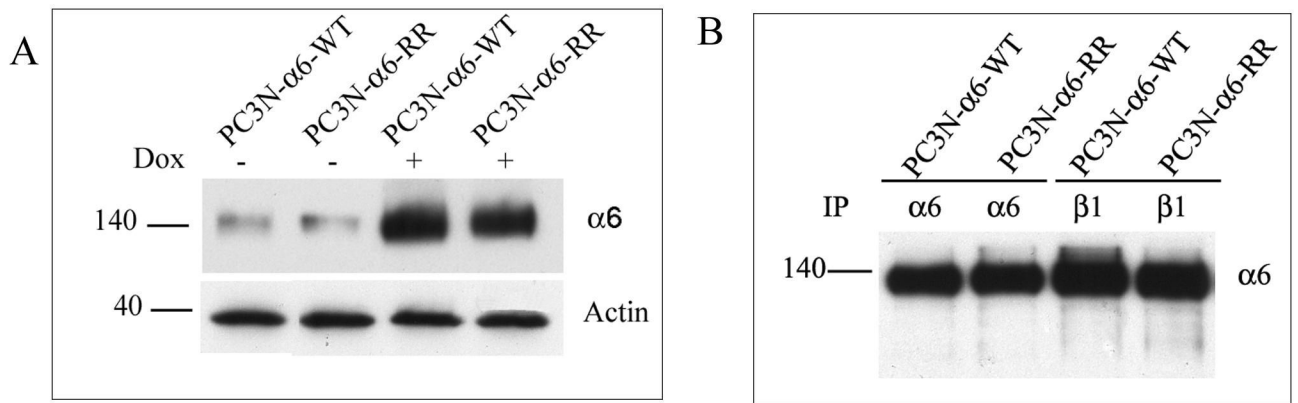
**Fig 2. Homology Model of the extracellular portion of the human integrin alpha 6 subunit (residues 24–1004) based on the crystal structure of the human alpha v beta 3 integrin (pdb: 1JV2) and sequence comparison of integrin  $\alpha 6$**

A. The first 23 residues in the integrin  $\alpha 6$  sequence undergo post-translational cleavage before the integrin reaches the surface and hence are not shown in the model. The model illustrates the location of the protease cleavage site within two consecutive arginine residues 594–595 (RR) shown along with their side chains. The beta propeller domain and a majority of the thigh domain (shown in white) are not contained in integrin  $\alpha 6p$  while the Calf-1 and Calf-2 domains of the stalk region remain (shown in black).

B. Comparison of the human integrin  $\alpha 6$  to the integrin  $\alpha 6$  sequence in mouse, rat, chicken and the integrin  $\alpha$  chain sequence of *C. elegans* and the S40311 integrin sequence of *D. melanogaster*.

C. Comparison of the human integrin  $\alpha 6$  to sequences of closely related integrins, integrin  $\alpha 7$  and integrin  $\alpha 3$ . The boxes indicate the R594R595 residues that are required for cleavage of integrin  $\alpha 6$  to integrin  $\alpha 6p$ . AliBee Multiple Alignment Release 2.0 was used to align the sequences.

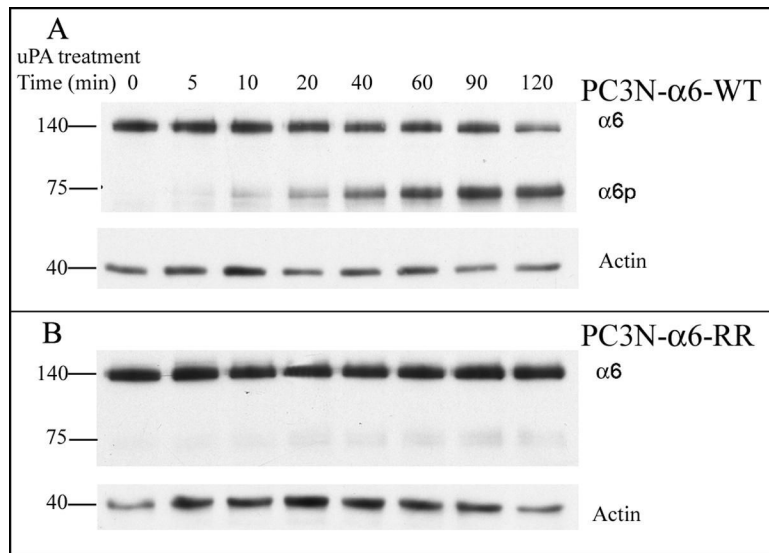




**Fig 3. Induction and expression of integrin  $\alpha 6$  heterodimers in transfected cells**

A. Cell lysates were analyzed for expression levels of integrin  $\alpha 6$  in PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR cells under unstimulated ((-) Dox) or stimulated (+) Dox conditions. The doxycycline concentration was 0.02 $\mu$ g/ml. The  $\alpha 6$  integrin was detected using the AA6A rabbit polyclonal antibody on a western blot. Actin was used as a loading control.

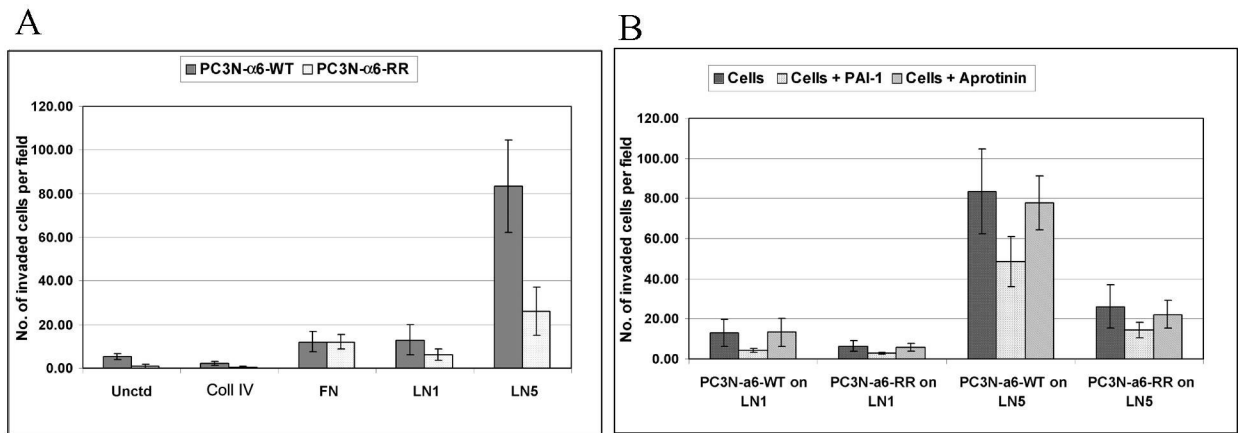
B. The wild type and mutated integrin  $\alpha 6$  binds to integrin  $\beta 1$ . Immunoprecipitations were done for integrin  $\alpha 6$  and integrin  $\beta 1$  in doxycycline (0.02 $\mu$ g/ml) induced PC3N- $\alpha 6$ -WT and PC3N- $\alpha 6$ -RR cells using rat anti-integrin  $\alpha 6$  antibody J1B5 and mouse anti-integrin  $\beta 1$  TS2/16. Rabbit anti-integrin  $\alpha 6$  antibody AA6A was used for western blotting.



**Fig 4. PC3N-α6-RR cells express the uPA non-cleavable form of the integrin α6**

A. PC3N-α6-WT cells were treated with 20 μg/ml uPA over time and the lysates analyzed by PAGE. The integrins α6 (140 kD) and α6p (~75 kD) were detected using the AA6A rabbit polyclonal antibody recognizing the α6 integrin cytoplasmic domain by western blot analysis. Actin (~40kD) was used as a loading control.

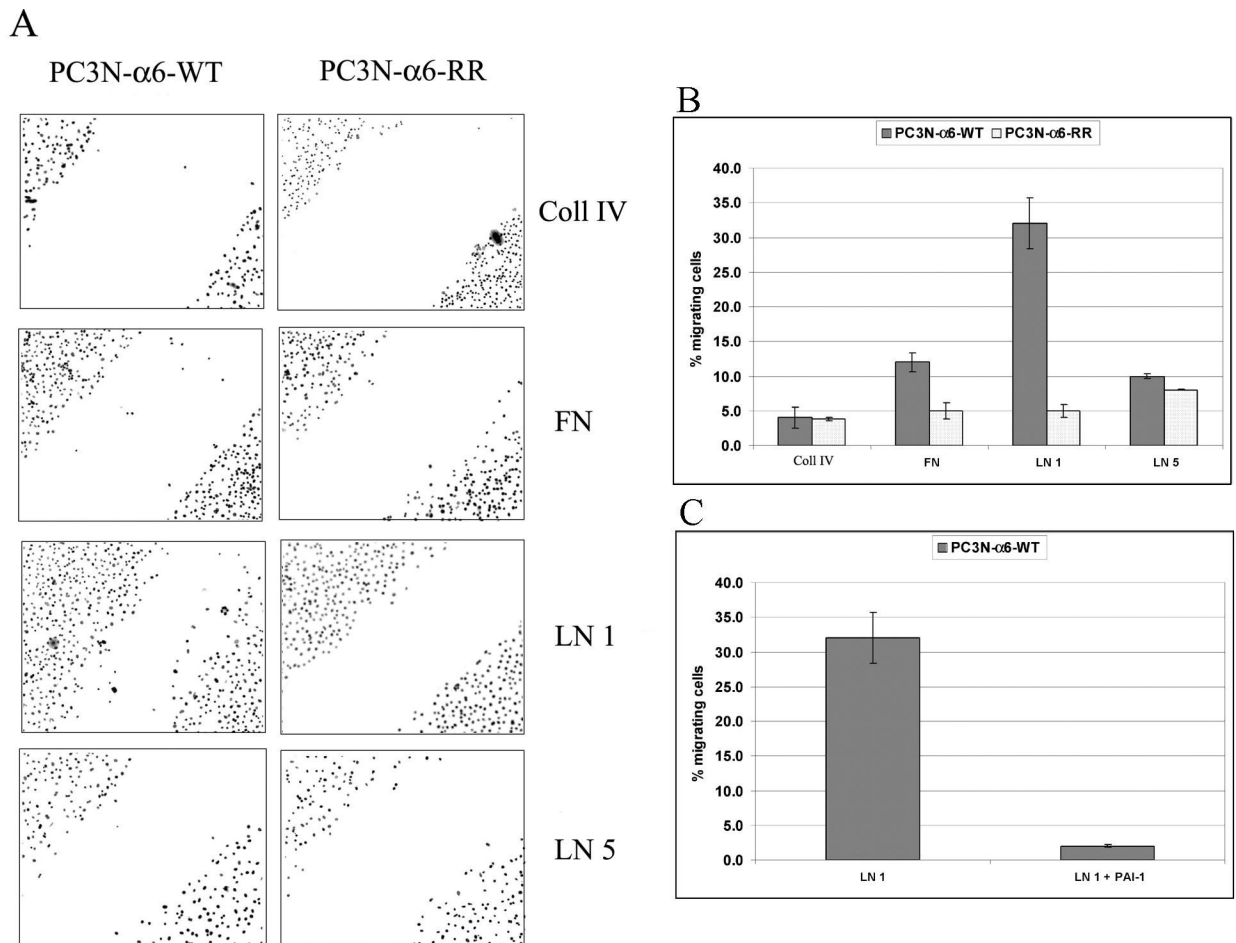
B. PC3N-α6-RR cells were treated with 20 μg/ml uPA over time and the lysates analyzed by PAGE. The integrins α6 (140 kD) was detected using the AA6A rabbit polyclonal antibody recognizing the α6 integrin cytoplasmic domain by western blot analysis. Actin (~40kD) was used as a loading control.



**Fig 5. Comparison of invasive potential of PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells. Doxycycline (0.02  $\mu$ g/ml) induced PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells were placed onto different ligands and incubated for 24 hrs at 37°C. 1 ng/ml EGF was placed in the bottom wells. The cells that had invaded the filters were stained with crystal violet and counted using 40X magnification. All experiments were done twice in triplicate**

A. Invasion of PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells through collagen IV (Coll IV), fibronectin (FN), laminin 111 (LN1) or laminin 332 (LN5) coated and uncoated transwell inserts.

B. Comparison of invasive potential of PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells through laminin 111 (LN1) or laminin 332 (LN5) coated transwell inserts in presence of uPA inhibitor PAI-1 (3  $\mu$ g/ml) and plasmin inhibitor aprotinin (10  $\mu$ g/ml).

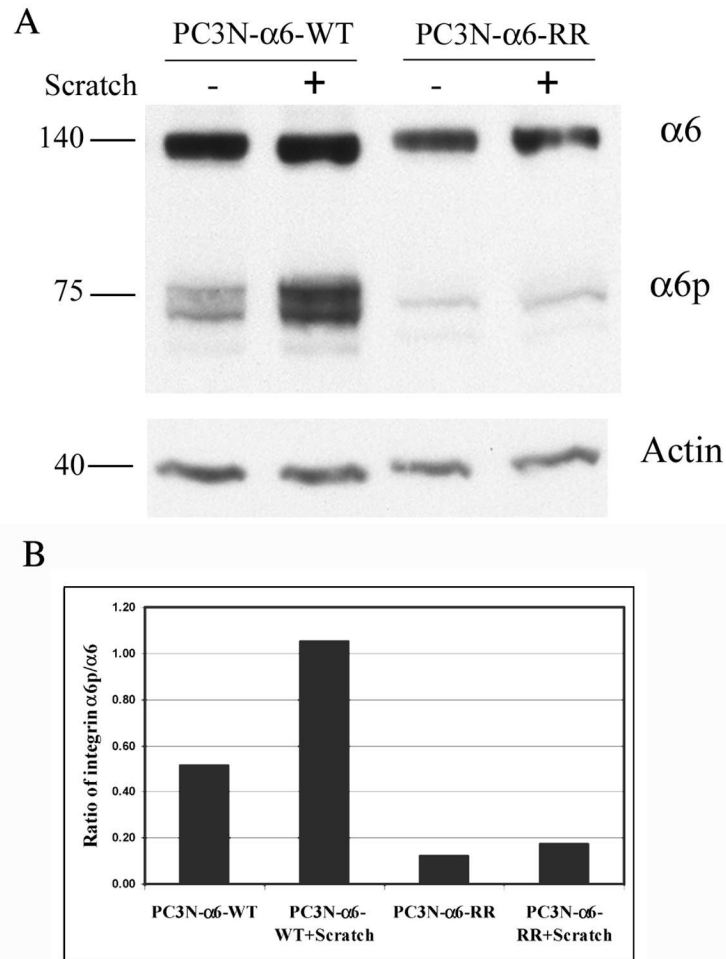


**Fig 6. Comparison of linear migration of PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells on different ligands.** PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells were grown to confluency on glass coverslips precoated with collagen IV (Coll IV), fibronectin (FN), laminin 111 (LN1) or laminin 332 (LN5). Migration was induced by scratching across the cell monolayer on the glass coverslip and addition of 1ng/ml EGF. The cells were fixed and stained with DAPI and incubated for 12 hours at 37°C. Images were collected using a Zeiss Axiovert microscope at 10 X magnification. The percentage of migrating cells was determined by using the particle counting feature of Scion Image software to detect the number of cells entering the scratch/total number of cells X 100. All experiments were done twice in triplicate

A. Digital images of PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells on collagen IV, fibronectin (FN), laminin 111 (LN1) and laminin 332 (LN5) 12 hours after scratching cell monolayer.

B. Comparison of percentage migration of PC3N- $\alpha$ 6-WT (solid bars) and PC3N- $\alpha$ 6-RR (open bars) cells on collagen IV, fibronectin (FN), laminin 111 (LN1) and laminin 332 (LN5), 12 hours after scratching cell monolayer.

C. Inhibition of linear migration of PC3N- $\alpha$ 6-WT by PAI-1 (3  $\mu$ g/ml) on laminin 111 (LN1).



**Fig 7. Induction of  $\alpha$ 6p in scratched PC3N- $\alpha$ 6-WT cells**

A. Doxycycline (0.02  $\mu$ g/ml) induced PC3N- $\alpha$ 6-WT and PC3N- $\alpha$ 6-RR cells were grown to confluency in a tissue culture dish, scratched and incubated overnight in fresh IMDM medium. The cells were then lysed and the integrin  $\alpha$ 6 detected using SDS-PAGE. The integrins  $\alpha$ 6 (~140 kD) and  $\alpha$ 6p (~75 kD) were detected using the rabbit antibody AA6A specific for the integrin  $\alpha$ 6 cytoplasmic domain. Actin (~40kD) was used as a loading control.

B. Densitometric analysis was done on the blot in Fig. 7A and ratios of the integrin  $\alpha$ 6p to full length integrin  $\alpha$ 6 were calculated and graphed.